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Effects of Brain Death and Hemodynamic Status on Function and Immunologic Activation of the Potential Donor Liver in the Rat

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Objective
To assess the effect on the function and immunologic status of potential donor livers of the duration of brain death combined with the presence and absence of hemodynamic instability in the donor.

Summary Background Data
Brain death, regarded as a given condition in organ transplantation, could have significant effects on the donor organ quality.

Methods
Brain death was induced in Wistar rats. Short or long periods of brain death in the presence or absence of hemodynamic instability were applied. Sham-operated rats served as controls. Organ function was studied by monitoring standard serum parameters. The inflammatory status of the liver was assessed by determining the immediate early gene products, the expression of cell adhesion molecules, and the influx of leukocytes in the liver.

Results
Progressive organ dysfunction was most pronounced in hemodynamically unstable brain-dead donors. Irrespective of hemodynamic status, a progressive inflammatory activation could be observed in brain-dead rats compared with controls.

Conclusions
Brain death causes progressive liver dysfunction, which is made worse by the coexistence of hemodynamic instability. Further, brain death activates the inflammatory status of the potential donor liver, irrespective of the presence of hypotension. The changes observed may predispose the graft to additional damage from ischemia and reperfusion in the transplant procedure.

Liver transplantation has evolved from an experimental operation in the 1970s to a standard treatment for end-stage liver disease today. There have been many achievements in organ transplantation in recent years, but major problems on the donor side remain, the shortage of donors being one. The lack of suitable donor organs and the increasing number of patients requiring an organ graft have resulted in the frequent acceptance of less-than-optimal donor livers for transplantation. These so-called marginal donor livers have a greater risk of primary dysfunction than livers retrieved from so-called optimal donors.

Organs with primary dysfunction have a significantly lower graft survival,[1,2] more acute rejection episodes, and a greater likelihood of developing chronic transplant dysfunction than organs with initially good function.[3–5] Primary dysfunction is associated with significant complications, an increased retransplantation rate, and death. Primary dysfunction can be caused by several factors, among which ischemia and reperfusion injury are of major importance. During the past few years, several groups have focused on

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the nonphysiologic state of brain death before organ retrieval as another potential risk factor for the outcome after transplantation.[6–8]

Brain death has been shown to have definite effects on hemodynamic stability,[9,10] hormone regulation,[11,12] and the inflammatory reactivity of the heart.[13,14] Only scarce information is available, however, on the relation between brain death and organs such as the donor liver.[6,7,15]

For this study, we developed a brain-death model in the rat that mimics either optimal (normotensive) or marginal (hypotensive) brain-dead donors. The model correlates well with the injury seen in human donors, where hemodynamic instability is inevitably encountered during the state of brain death unless appropriately treated.

With this rat model, we investigated the effects of brain death in normotensive and hypotensive donors. We studied the effects of brain death on the function and the inflammatory status of donor livers. Subsequently, we compared the quality of livers obtained from control rats with those of normotensive and hypotensive brain-dead rats, after short or long intervals after induction of brain death.

METHODS

All animals received care in compliance with the guidelines of the institutional animal ethics committee according to the Experiments on Animals Act (1996) issued by the Netherlands Ministry of Public Health, Welfare, and Sports.

Adult male Wistar rats (HSD.Cpb:WU, 300–350 g) were randomly allocated to six groups. In two control groups of six rats each, sham-operated rats served as non–brain-dead controls and were killed 1 or 6 hours after the onset of the experiment.

In the four groups of brain-dead rats, brain death was induced and the rats were killed 1 or 6 hours later. Two groups, the 1-hour and 6-hour optimal (normotensive) donors ($n = 6$ and $n = 10$, respectively), received donor management (hemodynamic support to achieve normotension). The two other brain-dead groups, the 1-hour and 6-hour marginal (hypotensive) donors ($n = 5$ and $n = 7$, respectively), did not receive donor management and remained hypotensive after brain-death induction.

Anesthesia and Ventilation

Halothane anesthesia was used in all procedures until the moment of brain-death induction. Rats were intubated and then ventilated using a Medec MK 78 infant ventilator (Medec NL, Wormerveer, The Netherlands). A mixture of nitrous oxide and oxygen was used for a period up to 30 minutes after brain-death induction (stroke rate 60 per minute, peak inspiratory pressure 12–14 mmHg, 40% inspiratory phase, 10% plateau). Subsequently, all rats were ventilated with 30% O_2 in air. The non–brain-dead control groups remained under anesthesia throughout the whole experiment.

Surgical Procedure

A laparotomy was performed through a midline incision for implantation of the telemetric device for continuous registration of mean arterial pressure (MAP) and heart rate (HR) (Chronic use TA11PA-c40 implant, Data Science Int., St. Paul, MN). The tip of the telemetry implant was subsequently inserted into the abdominal aorta just proximal to the bifurcation, after which the abdomen was closed.

Induction of Brain Death

Through a frontolateral trepanation (1×1 mm with a dental drill), a balloon catheter (Fogarty 14G, Baxter Health Care Corp., Irvine, CA) was introduced into the extradural space with the tip pointing caudally. Inflating the balloon for 1 minute increased the intracranial pressure, thereby inducing rapidly progressive brain injury and leading to immediate brain death. Initiation of brain death was defined by a sharp rise and then a subsequent drop of blood pressure and heart rate. The state of brain death was confirmed 30 minutes after induction of brain death by the absence of corneal reflexes and by an apnea test.

Donor Management

Rats in the optimal donor groups were kept normotensive by infusion of Gelofusin (Vifor Medical SA, Basel, Switzerland) in the tail vein at 0.5 mL per minute. A MAP of more than 80 mmHg was considered normotensive. In these two groups, treatment started 15 minutes after brain-death induction, allowing a period of hemodynamic stabilization after brain-death induction. If the infusion of Gelofusin was insufficient to maintain normotension, norepinephrine was added at a dose of 0.01 μm/mL. Animals in the marginal donor groups were not treated for hypotension during the whole study period. Control animals received 0.5 mL Gelofusin intravenously immediately after the sham operation.

Sampling Procedures

Just before termination of the experiment, serum was collected for biochemical analyses and liver tissue biopsies from the liver were taken at relaparotomy. Tissue samples were snap-frozen in isopentane (−80°C) or fixed in 4% paraformaldehyde.

Biochemical Determinations

Blood samples were analyzed in a routine fashion. Serum lactate dehydrogenase (LDH) and creatinine levels were used as indicators of general function of the donor. Lactate concentration was determined to identify the presence of acidosis. As indicators of liver damage, serum activities of aspartate transaminase (AST) and alanine aminotransferase (ALT) and the concentration of alpha-glutathione s-trans-

Table 1. PRIMARY ANTIBODIES USED FOR IMMUNOCYTOCHEMICAL ANALYSIS

Clone	Reactive With	Source
c-fos/AP-1	FOS	Calbiochem, Cambridge, MA
5F10	VCAM-1	Dr. R. Lobb, Cambridge, MA
1A29	ICAM-1	Dr. M. Miyasaka, Osaka, Japan
OX1	CD45	European Collection of Animal Cell Cultures
R73	TcR	Dr. Hunig, Wurzburg, Germany
OX8	CD8	European Collection of Animal Cell Cultures
0X35	CD4	European Collection of Animal Cell Cultures
HIS48	PMN	Dept. of Histology RUG, The Netherlands
NKR-P1A	NK cells	Pharmingen, San Diego, CA
ED1	Naïve MØ	Serotec, Amsterdam, The Netherlands
ED2	Active MØ	Serotec, Amsterdam, The Netherlands

ICAM = 1, intercellular adhesion molecule 1; NK, natural killer; PMN, polymorphonuclear cell; TcR, T-cell receptor; VCAM-1, vascular cell adhesion molecule 1.

ferase (α-GST) were used. The serum concentration of α-GST was measured with a quantitative enzyme immunoassay (Hepkit, Biotrin International, Dublin, Ireland). Total protein content of the serum was used to indicate hemoconcentration or hemodilution (Mega Merck, Darmstadt, Germany).

Immunocytochemistry of Liver Tissue

Changes in endothelial cell function and activation status are considered significant factors for success or failure of the graft.[16,17] Endothelial expression of cell adhesion molecules (intercellular adhesion molecule 1 [ICAM-1] and vascular cell adhesion molecule 1 [VCAM-1]) was studied in 6-μm sections of snap-frozen liver tissue. Activation of these cell adhesion molecules facilitates leukocyte recruitment. This leukocyte recruitment was analyzed by staining against CD45 (leukocyte common antigen). To detect nonspecific immune activation, leukocytes in the parenchyma were differentiated by staining against the T-cell receptor (TcR), CD8, and CD4 for detection of specific immune cell reactions and polymorphomononuclear cells (PMNs), macrophages, and natural killer cells (Table 1). After thorough washing, the sections were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark) and color-developed using 3-amino-9-ethylcarboxide (AEC)/H_2O_2. Sections were counterstained using Mayer's hematoxylin solution (Merck, Darmstadt, Germany). Routine hematoxylin and eosin staining was also performed on tissue sections of all rats.

Liver tissue was studied with respect to the expression of FOS, an immediate early gene product known to increase after stressful events. Samples of liver tissue fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4) were processed for staining of FOS (see Table 1). Samples were cryoprotected overnight in 20% sucrose. Serial 40-μm sections of the liver were collected in 0.02 mol/L potassium phosphate-buffered saline (pH 7.4) with azide and stored at 4°C until further immunocytochemical processing. Free-floating sections were incubated overnight in potassium phosphate-buffered saline with 2% normal rabbit serum, 2% bovine serum albumin, and sheep anti-FOS (1:2,000 diluted) in potassium phosphate-buffered saline/Triton 0.5% with 2% normal rabbit serum and 4% bovine serum albumin. Subsequently, the sections were incubated for 2 hours in rabbit anti-sheep IgG (1:800) in potassium phosphate-buffered saline/Triton 0.5% with 2% normal rabbit serum and 2% bovine serum albumin. Finally, the tissue sections were washed twice with sodium acetate 0.1 mol/L (pH 6.5) during 10 minutes before incubation with diaminobenzidine in 0.03 mL 1% H_2O_2. The reaction was stopped with sodium acetate 0.1 mol/L. Intermittent washing was performed with potassium phosphate-buffered saline.

Quantitation of immunocytochemical staining was assessed by light microscopy. FOS, VCAM-1, and ICAM-1 stainings were scored as negative (no staining) or weakly, moderately, or strongly positive. Microscopic examination and comparisons were always performed with biopsies simultaneously stained. Quantitation of stained cells in the sections stained for CD45, TcR, CD8, CD4, PMNs, macrophages, and natural killer cells was performed by counting the total number of positive cells per microscopic field at 400× magnification. Three people independently scored all samples.

Statistical Analysis

All results are expressed as mean ± standard error of the mean. Statistical analysis comparing different treatment modalities was performed using the Mann-Whitney test, with $P < .05$ considered significant. Statistical analysis comparing different time points was performed using the Kruskal-Wallis test, with $P < .05$ considered significant.

RESULTS

Effects of Brain Death and Donor Management on Blood Pressure and Heart Rate

The effects of brain death and donor management on the hemodynamic stability are shown in Figures 1 and 2. No differences between any of the groups in HR and MAP were observed before brain death. Mean HR and MAP were 372 ± 12 beats per minute (bpm) and 107 ± 3 mmHg, respectively. In the control groups, HR and MAP after the sham procedure were 383 ± 20.1 bpm and 99 ± 3.6 mmHg, respectively. In all rats exposed to brain-death induction, a steep rise in HR and MAP was observed, peaking at 472 ± 7.7 bpm and 162 ± 8.4 mmHg. Within 10 minutes of brain death, MAP decreased to a hypotensive level of less than 80 mmHg. The animals in both marginal donor groups re-

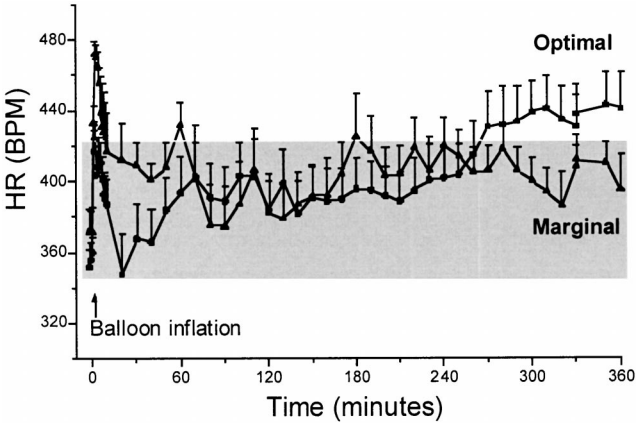

Figure 1. Changes in heart rate (HR, in beats per minute) after induction of brain death followed by up to 6 hours of normotension or hypotension in rats. HR is expressed as mean ± SEM. Normal range of HR in non–brain-dead control rats is shown by the shaded area.

ceived no Gelofusin infusion. They remained hypotensive, with mean blood pressures of 55 ± 5.3 mmHg after 1 hour and 52 ± 9.9 mmHg after 6 hours of brain death. HR in the marginal donor groups stabilized at a higher level than baseline. Before infusion therapy, no differences were seen in MAP or HR between the marginal and optimal donor groups. In the optimal donor groups, in all animals, normotension was achieved by fluid administration within 5 to 7 minutes of starting the Gelofusin infusion at 15 minutes after brain-death induction. This resulted in a significantly increased MAP compared with the marginal donor group. The MAP was 95 ± 2.7 mmHg after 1 hour of brain death and 88 ± 4.4 mmHg after 6 hours of brain death. The mean volume of Gelofusin infused was 3.3 ± 0.6 mL in the 1-hour optimal donor group and 10.1 ± 1.4 mL in the 6-hour optimal donor group. No norepinephrine administration was necessary to maintain normotension in the 1-hour brain-death group, but in the 6-hour optimal group, 7 of 10 rats needed additional support with norepinephrine infusion to remain normotensive.

Biochemical Determinations

Results of biochemical determinations are shown in Table 2. Serum parameters of LDH, AST, and ALT were corrected for hemodilution or hemoconcentration, as assessed by the total protein content of the serum. In non–brain-dead controls, the LDH activity as an indicator of general function was elevated at 1 hour after the sham surgical procedure but normal 6 hours after the sham surgical procedure. In marginal donors after brain-death induction, the LDH activity was elevated at 1 hour and increased to a higher level at 6 hours, indicating progressive lysis of cells. In optimal donors, similar activities of LDH were detected 1 hour after brain death. LDH activity remained elevated and was significantly higher 6 hours after brain-death induction than in controls. The increase in activity in marginal donors was significantly greater than in optimal donors. Also, serum creatinine concentration was significantly increased in the marginal donor groups ($P = .08$ after 1 hour, $P = .009$ after 6 hours) and in the optimal donor groups ($P = .03$ after 1 hour, $P = .04$ after 6 hours). Optimal donor groups also showed increased creatinine concentrations compared with controls, but only after 6 hours of brain death ($P = .02$). Lactate concentrations in marginal donors were significantly increased at all time points compared with optimal donors. Liver function parameter AST was significantly increased in the 1-hour and 6-hour marginal donor groups compared with controls ($P = .004$ and $P = .03$, respectively). In optimal donors, the change in AST activity was not significant ($P = .15$) (Table 2 and Fig. 3). In the 1-hour and 6-hour control groups, α-GST levels were within the normal range (Table 2 and Fig. 4). In marginal donor groups, after both 1 hour and 6 hours of brain death, significantly increased α-GST levels were found. Also, in 1-hour and 6-hour optimal donors, α-GST levels were significantly increased. All brain-dead groups showed a further increase in α-GST levels with time.

Immunocytochemistry of Liver Tissue

Table 3 shows the results of the immunocytochemical analyses. Photographs of examples of the expression of the ICAM-1, CD45, TcR, and PMN antigens are shown in Figure 5.

Adhesion Molecules

No VCAM-1 staining was observed in tissues of the control groups. In the brain-dead groups, however, VCAM-1 staining was seen on the endothelial cells of the

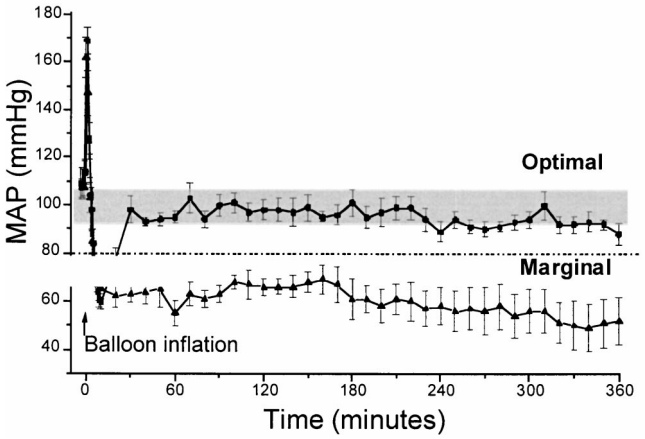

Figure 2. Changes in mean arterial pressure (MAP, in mmHg) in rats after induction of brain death. There is immediate hypertension followed by hypotension. Next, depending on the absence or presence of donor management, either hypotension persists (marginal donors) or normotension is achieved (optimal donors). MAP is expressed as mean ± SEM. The normal range of MAP in non–brain-dead control rats is shown by the shaded area. The cutoff point of hypotension (<80 mmHg) is represented by the dotted line.

Table 2. BIOCHEMICAL SERUM PARAMETERS

Parameter	Control (1 hour)	Control (6 hours)	Marginal (1 hour)	Marginal (6 hours)	Optimal (1 hour)	Optimal (6 hours)
Na^+ (mmol/L)	138 ± 0.5	140 ± 0.7*	139 ± 2.2	139 ± 1.6‡	140 ± 0.9	145 ± 1.7*†
K (mmol/L)	5.2 ± 0.2	4.9 ± 0.2	6.3 ± 0.4	9.6 ± 1.2*†‡	5.9 ± 0.3	5.9 ± 0.5
LDH (IU/L)	1589 ± 240	410 ± 82*	1357 ± 350	3037 ± 1008†	1603 ± 256	1660 ± 421†
Creatinine (μmol/L)	59 ± 2.6	43 ± 2.2*	87 ± 3.1†‡	178 ± 37*†‡	60 ± 5.8	98 ± 25†
Lactate (mmol/L)	3.4 ± 0.4	1.2 ± 0.1	4.7 ± 0.7‡	4.9 ± 1.1†‡	2.2 ± 0.7	2.2 ± 0.8
AST (IU/L)	93 ± 17	134 ± 28	253 ± 49†‡	272 ± 46†‡	129 ± 13	151 ± 27
ALT (IU/L)	50 ± 3.2	52 ± 4.4	42 ± 1.9	59 ± 16.3	55 ± 6.9	39 ± 5.0
α-GST (μg/L)	25 ± 3.8	18 ± 11	428 ± 111†	919 ± 219†	168 ± 40†	642 ± 178†

ALT, alanine aminotransferase; AST, aspartate transaminase; α-GST, alpha-glutathione s-transferase; LDH, lactate dehydrogenase.
Values are mean ± SEM.
Serum parameters LDH, AST, and ALT are corrected for hemodilution or hemoconcentration as assessed by the total protein content of the serum.
$P < .05$ vs. * 1 hour, † controls, and ‡ optimal donors.

central veins after 6 hours of brain death, but not after 1 hour. ICAM-1 was found to be constitutively expressed on sinusoidal endothelium and the central vein endothelial cell lining of the liver. Compared with the constitutive staining in the control groups, expression of ICAM-1 was increased to a similar extent in both marginal and optimal donor groups, with an intensity progressing with duration of brain death.

Leukocyte Infiltration

In 1-hour and 6-hour controls, a baseline level of leukocyte infiltration (i.e., CD45-positive cells) was observed in liver tissue (see Table 3 and Fig. 5). In the marginal donor groups, the number of infiltrating leukocytes was already significantly increased 1 hour after induction of brain death, and the accumulation increased with duration of brain death. In the optimal donor groups, a similar increase in the amount of CD45-positive cells was observed. Of these leukocytes, some were found to be T lymphocytes (i.e., TcR-positive cells). In marginal donors, 1 hour after brain-death induction, the amount of T lymphocytes was small but significantly increased when compared with controls. Six hours after induction of brain death, the T-lymphocyte count was higher than at 1 hour. In optimal donors, there was no significant increase in T lymphocytes after 1 hour of brain death. After 6 hours of brain death, a significant increase in the amount of T lymphocytes was observed ($P = .03$), although this increase was not as pronounced as in marginal donors. The number of CD8-positive lymphocytes was significantly increased in 1-hour marginal donors compared with controls and optimal donors. No differences between control, marginal, and optimal donors were observed after 6 hours of brain death. In all groups, the number of CD8-positive cells increased with time. CD4-positive lymphocytes showed no significant differences between any group or time.

The number of PMNs (i.e., HIS 48-positive cells) in controls increased slightly during the experiment. In marginal donors, however, a significant increased influx of PMNs was observed, an effect that was progressive with the duration of brain death. In optimal donor groups, the amount of infiltrating PMNs progressively increased. The correlation index between the leukocyte and PMN recruitment was 0.88 (RSQ), indicating that the increase in leukocytes in liver tissue consisted mainly of PMNs. The number of natural killer cells in the liver parenchyma remained small in all

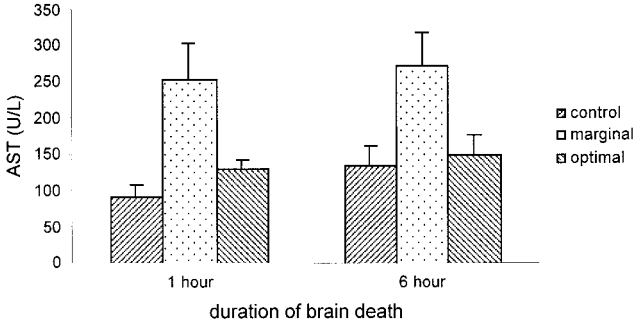

Figure 3. Aspartate transaminase (AST) activity reflects liver dysfunction in non–brain-dead control rats and marginal and optimal brain-dead donors 1 and 6 hours after brain-death induction. Results are expressed as mean ± SEM.

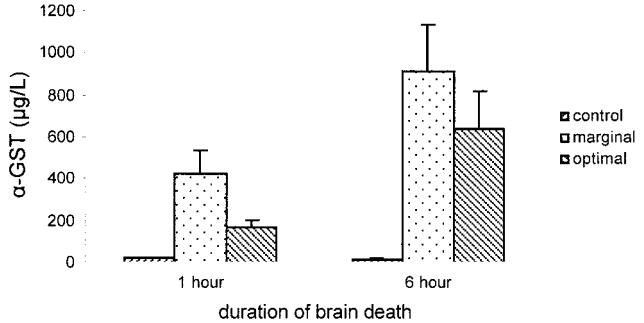

Figure 4. Alpha-glutathione s-transferase (α-GST) concentration reflects liver dysfunction in non–brain-dead control rats and marginal and optimal brain-dead donors after 1 and 6 hours. Results are expressed as mean ± SEM.

Table 3. IMMUNOCYTOCHEMICAL ANALYSIS

	Control (1 hour)	Control (6 hours)	Marginal (1 hour)	Marginal (6 hours)	Optimal (1 hour)	Optimal (6 hours)
FOS	−	−	+	+	−	−
VCAM-1	−	−	−	+	−	+
ICAM-1	+	+/++	+/++	+/+++	+	+/+++
CD45	7.9 ± 1.1	12.3 ± 0.6	13 ± 1.1†	24 ± 4*†	17 ± 2.6†	23 ± 2†
TcR	1.1 ± 0.1	0.7 ± 0.1	2.6 ± 0.4†	3.6 ± 1†	2 ± 0.5	1.5 ± 0.2†
CD8	1.2 ± 0.2	3.8 ± 0.5*	3.2 ± 0.4†‡	6 ± 1.1*	1.9 ± 0.3	3.9 ± 0.4*
CD4	0.3 ± 0.1	1.4 ± 0.5	1.1 ± 0.7	2 ± 0.5	1.7 ± 0.4†	1.2 ± 0.2
PMN	4.1 ± 0.5	6.2 ± 0.8*	9.7 ± 0.9†	21.7 ± 4*†	13.2 ± 1.8†	27.7 ± 3.1*†
NK cells	0.74 ± 0.3	0.78 ± 0.5	0.51 ± 0.2	1.49 ± 0.6	0.58 ± 0.2	1.22 ± 0.5
ED1	19 ± 0.4	26 ± 6.4	34 ± 5.7†	35 ± 3	27 ± 1.8†	31 ± 1.9
ED2	16 ± 1	19 ± 3.2	26 ± 2.5†	25 ± 1.8	20 ± 0.8†	22 ± 1.1

ICAM-1, intercellular adhesion molecule 1; NK, natural killer; PMN, polymorphonuclear cell; TcR, T-cell receptor; VCAM-1, vascular cell adhesion molecule 1.
−, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.
Values are mean number of antigen-positive cells per microscopic field at 400× magnification ± SEM.
$P < .05$ vs. * 1 hour, † controls, and ‡ optimal donors.

groups, with no significant differences. In contrast to the small number of natural killer cells, large numbers of macrophages were found in liver tissue. In marginal and optimal donor groups, the number of naïve macrophages (i.e., ED1-positive cells) was increased ($P < .01$) after 1 hour of brain death compared with controls. After 6 hours of brain death, the pattern was similar to that found after 1 hour of brain death. The number of activated macrophages (i.e., ED2-

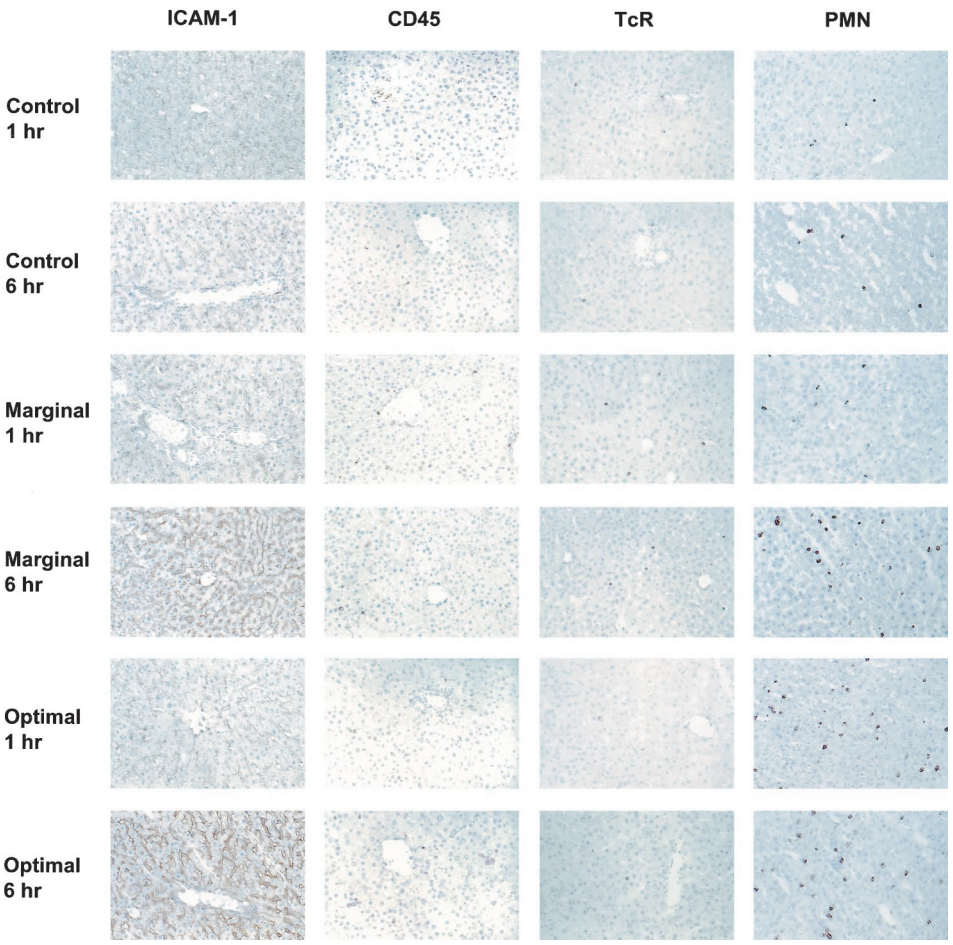

Figure 5. Microscopic analysis of immunocytochemical stains of relevant primary antibodies in liver tissue of non–brain-dead control rats and marginal and optimal brain-dead donors after 1 and 6 hours.

positive cells) showed a pattern similar to the ED1 staining, but the absolute numbers were smaller.

Immediate Early Genes

No expression of FOS was observed in non—brain-dead controls or in normotensive optimal brain-dead donor livers at any of the time points tested. FOS expression in the liver was observed in the 1-hour and 6-hour marginal brain-dead rats only. The number of areas expressing FOS increased with duration of brain death. Expression was found primarily in areas surrounding the central veins (Rappaport zone III).

DISCUSSION

There have been only a few studies of the effects of brain death on donor organ quality. These have focused on donor hearts because of direct pathophysiologic aspects in the heart muscle related to brain death. We have previously demonstrated in a brain-dead rat model that the donor liver suffers from the state of brain death. In this report we show, for the first time, how the potential donor liver is affected by brain death with subsequent normotension compared with continuing hypotension. Because of the persisting imbalance between the number of donor organs needed and the number available for transplantation, marginal donor livers are more and more frequently accepted today, despite the increased risk of these organs to develop primary dysfunction. These results should increase our knowledge of brain death and organ preservation in general and our understanding of using livers from hypotensive marginal donors in particular. This could help us to design interventions that aim to reduce damage and improve posttransplant function, thus allowing an increase of the donor pool.

In this report, we demonstrate a time-dependent progressive organ dysfunction before organ retrieval and preservation that is most pronounced in marginal donors. Simultaneously, there is an increased immune activation with expression of cell adhesion molecules. This effect coincides with leukocyte infiltration in the donor liver parenchyma, irrespective of the hemodynamic stability of the donor.

Effects of Brain Death on Liver Dysfunction

Brain death in the donor causes time-dependent general dysfunction, as indicated by elevated LDH and creatinine levels in all brain-death groups. In addition, progressive liver cell dysfunction is induced by the sequence of events resulting from brain death. This was shown by the increased levels of AST and α-GST. Achieving normotension after inducing brain death, as in the optimal donor groups, reduced the severity of the specific liver cell dysfunction, but it did not fully prevent the other dysfunction related to brain death.

An effect of hypotension on liver function is to be expected, because studies have shown decreased liver function as a result of hypovolemic vascular shock. Hypotension is common after brain death, as shown by both animal and human studies.[7,10,18,19] We restored blood pressure by Gelofusin infusion, which has been demonstrated to be better than saline infusion (data not shown), to compensate for any eventual effects of hypotension during brain death. Norepinephrine was found to be effective to maintain normotension when Gelofusin infusion was insufficient. The effects of norepinephrine mainly involve MAP, without a dramatic increase in HR, as demonstrated in humans by Iwai et al.[20,21] In contrast, in this model, both epinephrine and dopamine had greater effects on HR, leading to tachycardia and eventually to cardiac failure (data not shown).

Unlike AST, there were no significant changes in ALT activity in any of the groups. In an ischemia–reperfusion model of the liver in the rat, van Wagensveld et al.[22] reported unchanged ALT activity despite liver cell dysfunction evidenced by increased levels of AST and α-GST.

Effects of Brain Death on Immunologic Status of the Liver

FOS expression was examined to investigate whether brain death would induce a cellular stress response in liver tissue. FOS expression was found only in hypotensive brain-dead rats, indicating that hypotension, rather than brain death, triggered the cellular stress response that initiates FOS production.

Increased cell adhesion molecule expression on liver parenchyma was observed, irrespective of the hemodynamic stability of the donor. VCAM-1 expression was found after 6 hours of brain death in both marginal and optimal donors. The pattern of VCAM-1 expression was similar to that described by others during periods of inflammation or rejection of the liver.[23] Also, the ICAM-1 expression observed in all brain-dead rats was similar to expression patterns during episodes of inflammation.[24–27] During fulminant inflammation, VCAM-1 and ICAM-1 expression by other cell types at other locations in the liver has been reported,[25,27,28] suggesting that different regulatory pathways may be involved during brain death.

Leukocyte recruitment to the underlying parenchyma was facilitated, as expected, with upregulated VCAM-1 and ICAM-1 expression, with a significant increase in infiltrating leukocytes (i.e., CD45-positive cells) in the liver tissue of all brain-dead rats. The number of leukocytes increased in virtually the same numbers with duration of brain death, irrespective of the donor regimen. T lymphocytes (i.e., TcR-positive cells) represented a small fraction of recruited leukocytes, whose numbers were different in optimal and marginal donor groups ($P = .06$), unlike the cells revealed by the other antibody stains. This suggests that hemodynamically unstable donors have a more profound T-lymphocyte recruitment. A substantial proportion of the increased number of leukocytes consisted of cells of the nonspecific

immune response, primarily PMNs. Also, naïve as well as activated macrophages (i.e., ED1- and ED2-positive cells) were significantly increased in brain-dead donors versus controls.

Recently, Koo et al[29] observed a similar activation of adhesion molecules in human cadaveric donor kidneys before reperfusion, compared with kidneys from living related donors. In contrast to their observation, we found a significant influx of PMNs into the parenchyma of the liver after the activation of the adhesion molecules. Whether this difference is due to preservation, is organ-specific (kidney vs. liver), or is related to the duration of brain death is not known.

The immune activation observed in our study resembles an innate immune response, with an induced expression of adhesion molecules on endothelium that recruits circulating monocytes/macrophages and PMNs. The increase in T lymphocytes, however, corresponds with a specific immune response. The small number of T lymphocytes relative to the number of PMNs could arise if the 6-hour period of observation after induction of brain death were too short. An important role of T-lymphocyte activation in the immune modulation after prolonged brain death cannot be excluded. This has been suggested by Takada et al[6] after examination of kidney tissue. Whether this specific immune activation plays a role in subsequent organ damage must be confirmed in a transplantation model. Possibly, the specific immune activation of T lymphocytes is caused by a danger signal, as proposed by Matzinger.[30] This danger theory states that the immune system can react to a nonspecific immune stimulus with a specific immune response when this stimulus is interpreted as being dangerous. The sequence of events induced by brain death, with alterations in neurohumoral release, hemodynamic profile, metabolism, and cytokine expression, could represent such a stimulus.

In addition to the activation of the potential donor liver, providing a foothold for the immune response of the recipient, which can lead to an increased immune response after transplantation, the influx of PMNs can have an additional detrimental effect. Neutrophils can act as mediators of tissue destruction by mechanisms such as their production of oxygen radicals and various proteinases.[31] The influx of these cells in the liver tissue of brain-dead donors may predispose these organs to cell destruction, with loss of donor organ quality on transplantation. This could explain in part why in kidney transplantation, living (un)related donor organs have consistently higher graft survival and negligible rates of primary dysfunction compared with organs retrieved from a cadaveric donor.[32,33]

The agonal phase in the presence of brain death induces a cascade of events that leads to a significant progressive dysfunction of the liver and a progressive immune cell activation. To a certain extent the dysfunction can be reduced by maintaining normotension in brain-dead donors. Better donor management may also diminish the influx of T lymphocytes, but it will not have a beneficial effect on the immune-activated state of potential donor organs. Progressive dysfunction of the liver and infiltration of neutrophils with increased immunogenicity of the potential donor organ decrease organ viability and, at the time of transplantation, may lead to more primary dysfunction, acute rejection, and lower graft survival.

We believe that the state of brain death should no longer be considered as a fixed and static condition, but a dynamic process that directly influences donor organ quality. The hours between declaration of brain death and organ retrieval might give us the opportunity for cytoprotective intervention and thus counteract or potentially diminish the detrimental effects of brain death. This could further improve donor organ quality, resulting in fewer complications, increased survival, and most importantly a better long-term function of the cadaveric donor organ.

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Discussion

PROF. P. BELL (Leicester, United Kingdom): This is an interesting paper. I think you got a bit confused between organ damage due to lack of perfusion and the immune response. You did not mention Tilney's paper from 1998, which was published in *Transplantation* and showed that with this kind of model, which he called a cytokine bomb, you get upregulation of a number of inflammatory agents in the kidney, the liver, and so on. You can abolish this by giving antagonists. He has already done that work and you did not mention it. Could you comment on this, please? And could you also tell me the evidence that people who have organs that are damaged by ischemia do worse? In our practice, they do as well as the patients who have kidneys from so-called heart-beating donors. Would it not be better to separate these two situations and compare organs from brain-dead rats or humans with donors who have a sudden death (non–heart-beating donors) because they do not have time to upregulate cytokines? Surely the best model is to take organs from a non–heart-beating human donor and put that kidney onto a machine to see if that organ can be resuscitated and then transplanted. We are already doing a project sampling tissue from livers, kidneys, and other organs in these donors to see whether or not there is upregulation of cytokines.

DR. R. PLOEG (Groningen, The Netherlands): I will leave it to the audience to decide who is confused. We are very well aware of Nick Tilney's group, with whom we communicate, and in our discussion, we actually refer to his group's 1998 *Transplantation* paper by Takada. As we could confirm in our previous paper (*Transplantation* December 1999), Takada showed an activation of proinflammatory mediators in the hypotensive brain-dead rat model. He has focused on immunohistological staining for a number of cell types and cell surface molecules in the kidney, and on PCR assays to demonstrate upregulation of a number of cytokines in other organs. He then showed that a soluble P-selectin ligand or T-cell activation blockade with $CTLA_4$ Ig suppressed macrophage and T-cell–associated products. This, however, does not imply, as the authors state themselves, that all effects related to brain death are abolished. To quote the authors, "the observed effects are the result of massive acute central injury, hypotension, and circulation factors." To unravel the effects of brain death and hemodynamic instability, we have developed a hypotensive (marginal) and a normotensive (optimal) brain-dead donor rat model and compared groups to controls. We could show for the first time in the liver, and also in the kidney, a significant and progressive activation of a nonspecific immune response irrespective of hemodynamic stability.

Concerning your second question, in general, warm and cold ischemia are both known to cause injury to an organ, although the number of minutes or hours leading to irreversible dysfunction may vary by organ and species. A good example for the effects of ischemic injury in transplantation is the increased incidence of the delayed graft function and higher chance of acute rejection, followed by a lower graft survival in kidney transplantation. This has been shown by several groups, including our own. In this respect, you are referring to Kootstra's work: he and others demonstrated a significantly higher percentage of delayed graft function (80%) in kidneys from non–heart-beating donors compared to those from heart-beating donors (30%). In the limited numbers he evaluated, as of today, no effect on short-term graft survival has been seen after the kidneys have started to function. In other studies, however, it is shown that the occurrence of delayed graft function has a significant effect on chronic transplant dysfunction and graft survival (*Transplantation* 1992;53:323).

I agree with you that a comparison of effects between heart-beating and non–heart-beating donors on potential organs is important. We obviously had the same idea as you, and started a clinical project comparing different types of cadaveric donors, using living donors as controls. We are anxious to see whether less

activation after a sudden death in the non–heart-beating donor situation, but more ischemic injury, will outweigh the detrimental effects of a period of brain death in the heart-beating donor.

PROF. P. KINNAERT (Brussels, Belgium): Dr. Ploeg, your data are important for people involved in organ retrieval and organ transplantation. However, they raise many questions. Because of time limitations, I will have to restrict myself to two topics. In your so-called optimally treated rats, you monitored the mean blood pressure and prevented its drop by plasma expansion and norepinephrine, but you still observed a significant increase in blood creatinine levels. You attribute this finding to the cerebral trauma. A normal blood pressure does not mean that the organs are correctly perfused and that the peripheral oxygen delivery is normal. Have you any data showing that organ blood flow and oxygenation of tissues were normal in your model? Indeed, your data concerning adhesion molecules are reminiscent of the effect of hypoxia on endothelial monolayers. Hypoxia also induces an increase of the expression of adhesion molecules (selectins, ICAM-1) by these cells. Therefore, one wonders whether your findings are due to cerebral trauma or to tissue hypoxia. You administered a rather large volume of plasma expander in normovolemic rats, which could induce oxygen diffusion problems in their lungs. How were the lungs of these animals?

Concerning the immunocytochemical studies, have you any idea of the sensitivity of the labeling methods? For instance, which kind of TcR did the monoclonal antibody detect? I ask this question because when you add up the CD4 and CD8 T cells, you sometimes end up with a much higher number of cells than the number labeled by the antibody against the TcR. Similarly, are the cells labeled with the ED1 and ED2 antibodies only infiltrating monocytes, or do these antibodies also recognize Kupffer cells? Indeed, the number of cells labeled by both antibodies is greater than the total number of CD45-positive cells. I also have questions related to the specificity of the immune response and to the immunogenicity of the graft, but we can discuss them later.

DR. PLOEG: Prof. Kinnaert, in our normotensive (optimal) brain-dead donors, we have checked the oxygenation of tissues and found serum pO_2, pCO_2, HCO_3^-, pH, and lactate levels to be sufficient. No differences with controls ventilated for 6 hours were observed. As far as plasma expansion is concerned, we have administered a mean of 9 mL Gelofusin to achieve and maintain normotension (<30% of blood volume). No oxygen diffusion problems were found in any of the rats, confirming previous findings by Novitzky et al. You also wondered which kind of T-cell receptor did the monoclonal antibody detect, because when CD4- and CD8-positive T cells are added up, the number of cells together is higher than the antibody labeling for TcR. We think that this might be due to difficulties in the TcR staining, which shows a halo around positive cells that is easier to miss than a positive staining for CD8 and CD4. Even when CD8- and CD4-positive cells are added up, however, there is no significant difference with the total numbers of TcR-positive staining cells. ED1- and ED2-positive cells should not be added up, as there is a wide overlap of the cells stained by these primary antibodies (i.e., Kupffer cells). It is the difference in qualitative staining that is more indicative of activation.

PROF. P. MORRIS (Oxford, United Kingdom): I want to make one comment and then ask a question. The first is that you mentioned that living unrelated donor and recipient kidney transplants do as well as living related donors who differ at one haplotype for HLA, such as parent to child or siblings sharing one haplotype. That is true overall, but I should point out, now that there is a much larger experience available, there is the same hierarchy of matching of living unrelated donors and recipients as there is in cadaver transplantation. In other words, a badly matched husband and wife do very much worse than husband and wife who were well matched by chance, which is perhaps not surprising.

The other point I want to make is that you probably know that Sue Fuggle and Dicken Koo in my unit have been studying biopsies of human kidneys, both from cadavers and living related donors, before and after transplantation, as well as after brain death. They have been able to show in cadaver kidneys following brain death—but never in living related kidneys—upregulation or expression of HLA class 2, ICAM, and VCAM on endothelium before the kidney is implanted. When it is biopsied 30 minutes later, there is a significant infiltration of neutrophils in about 50% of cadaver kidneys—but again, never in living related kidneys. This we attribute to ischemia-reperfusion injury, but in your study you have seen this quite significant infiltration of neutrophils in association with brain death, which we have not seen in the human kidney. So there is a disparity there, and I just wonder if you have any explanation for that. That is rather a tricky question, I realize, because I do not have one either, off the top of my head. Your study is very important, and I hope you will continue this, for the whole question of the impact of brain death on the quality of organs to be transplanted is becoming of increasing relevance to graft outcome.

DR. PLOEG (Closing Discussion): I do agree with your remark that better matching in living unrelated donor–recipient combinations will undoubtedly have an effect on a better outcome after transplantation. I have enjoyed the paper by Drs. Koo and Fuggle very much; however, I have no explanation for the different observations. Koo et al from your institution showed no influx of PMNs after cold ischemia and prior to reperfusion, while we definitely demonstrated a very positive PMN staining after 6 hours of normotensive brain death, even without preservation. Duration of brain death could be speculated. We feel that in a short-lived animal such as the rat, the type of injury is severe, and a time span of 6 hours of brain death is long. We are currently investigating the effect of duration of brain death in our human donors by looking at endothelial activation, influx of different cell types into the parenchyma, and percentage of apoptosis.